Dimethylarginine dimethylaminohydrolase-1 is involved in spinal nociceptive plasticity

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INTRODUCTION

Activation of neuronal nitric oxide synthase (nNOS), and consequent generation of nitric oxide (NO), is coupled to upstream stimulation of *N*-methyl-D-aspartate (NMDA) receptors [9-11]. In the spinal dorsal horn, NO contributes to sensitization of nociceptive signalling pathways in both acute and chronic pain states, though it is not thought to be involved in basal pain perception [12; 15; 22; 20; 4; 11; 30].

A key endogenous inhibitor of NOS is asymmetric dimethylarginine (ADMA), which is liberated following post-translational methylation of arginine residues within proteins, and subsequent proteolysis [14] [21]. Structurally similar to L-arginine, ADMA competes for the active site of NOS and thus regulates NO formation. Physiological concentrations of ADMA in the brain are sufficient to modulate nNOS function and suppress NO-mediated excitotoxicity [3]. Other evidence suggests that ADMA modulates various processes in the central nervous system (CNS), including the response to nerve injury and in nociception [25; 14]. ADMA itself is actively regulated by dimethylarginine dimethylaminohydrolase (DDAH), of which there are two isoforms, DDAH-1 and DDAH-2; this supports the concept that ADMA confers an important physiological function [18; 32]. DDAH metabolises ADMA to L-citrulline and dimethylamine, regulating the inhibitory influence of ADMA on NOS (Supplementary Figure 1A). Accordingly, pharmacological inhibition of DDAH increases levels of ADMA sufficiently to attenuate NO synthesis [29; 17]

We have developed a competitive inhibitor of DDAH, N^G-[2-methoxyethyl] arginine methyl ester (L-291) [29], selective for DDAH-1 [26]. L-291 indirectly reduces NO signalling through accumulation of ADMA [29; 17; 26]. Inhibition of DDAH-1 within the cardiovascular system was found to be protective in rodent models of septic shock, in which NO reaches pathological levels and contributes to sustained circulatory collapse [17; 26; 34]. Targeting

DDAH isoforms may be beneficial in other pathologies in which increased NO signalling is implicated, particularly within the CNS.

The distribution of DDAH mRNA correlates well with NOS expression in a number of tissues; in fact, DDAH-1 is found predominantly in tissues expressing nNOS [18; 32]. Both DDAH and nNOS mRNA are upregulated in axotomized motoneurons [25]. The functional contribution of DDAH in the nervous system, however, remains largely unknown. Here, we have investigated the role of DDAH-1 in the nervous system, specifically in the spinal cord, by administering L-291 in two models of spinal nociceptive plasticity.

MATERIALS AND METHODS

Animals

Experimental procedures in adult male Sprague-Dawley rats (220-250 g) or C57BL/6 mice (~25-30g), Central Biological Services - University College London, King's College London or Harlan, UK were approved by the UK Home Office and were conducted in accordance with the guidelines of the International Association for the Study of Pain [35].

DDAH-1 specific inhibitor, L-291 and control inactive enantiomer, L-456

L-291 (N^G-[2-methoxyethyl] arginine methyl ester; Supplementary Figure 1b is a selective competitive inhibitor of DDAH-1 while L-456 is its inactive D-enantiomer and serves as a control). Both compounds were dissolved in 0.9% saline. Full details of their synthesis and characterization have been described previously [29; 17].

DRG culture and fluorometric detection of nitrite and ADMA

DRG from adult male rodents were removed into sterile Ham's F12 medium (Gibco 21765-029) and DRG neurons were isolated as described previously [27; 1]. Neurons were plated into 96-well plates, coated with poly-L-lysine (100 µg/ml; Sigma P8920) and laminin (10 µg/ml; Sigma L-2020). After 24 hrs the medium was replaced with customised (100 µM L-arginine) Dulbecco's Modified Eagle Medium (Gibco) containing L-291 (0 – 10 mM) for a further 24 hours, after which media was acquired for either nitrite or ADMA measurement.

For nitrite measurements, 100 µl medium was removed from each well and transferred to a black-bottomed 96-well plate. Nitrite levels were measured by 2,3-diaminonaphthalene (DAN) fluorometry, as per Misko *et al.* [23]. Fluorescence was recorded using a spectrofluorometer equipped with Xenon lamp source (excitation 365 nm; emission 410 nm). Nitrite levels were calculated from linear calibration curves of known nitrite concentrations (0 – 5 µM) made up in the same medium.

Methylarginines (ADMA and SDMA) were measured from the cell culture media using LC-MS/MS as previously described [2].

Western immunoblotting of DDAH-1 in neuronal tissues

Urethane-anesthetized rats were sacrificed by decapitation and tissues (spinal dorsal horn, DRG and hippocampus) were dissected and snap frozen. Tissues were homogenized in RIPA (radioimmunoprecipitation assay) buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS + 0.5% deoxycholic acid + complete protease inhibitor cocktail) using a glass homogenizer. Homogenates were centrifuged at 14000 rpm for 10 min at 4°C and supernatants containing whole cell tissue lysates were collected. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Cramlington, UK). Laemmli loading buffer was added to protein lysates (40 μg) and samples were incubated at 70°C for 30 min, before loading onto 8% gels, and separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Proteins were transferred to nitrocellulose membranes and probed overnight at 4°C with goat anti-DDAH-1 (1:500; ab2231, Abcam, Cambridge, UK) or rabbit anti-neuronal β-III Tubulin (1:3000; ab18207, Abcam), which served as a loading control. Membranes were incubated with IRDye-linked donkey anti-goat 680 or donkey anti-rabbit 800CW secondary antibody (1:15000) for 1 hr at RT. Proteins were revealed using the Odyssey fluorescence detection system (Licor, Cambridge, UK).

Immunohistochemical detection of DDAH-1

Rats were anaesthetized using sodium pentobarbital (60 mg/kg) and perfused transcardially with 100 ml of saline followed by 500 ml of 4% paraformaldehyde (PFA) in 0.2 M phosphate buffer pH 7.4 (PB). The spinal cord and lumbar L5 DRG were dissected and post-fixed at 4°C overnight (spinal cord) or for 2 hrs (DRG). Tissues were cryoprotected overnight at 4°C in

30% sucrose in 0.1 M phosphate buffered saline (PBS), and then frozen in O.C.T. compound. DRG sections (20 µm) were cut using a cryostat and collected directly on slides; spinal cord sections (30 µm) were collected in PBS for 'free-floating' immunostaining.

All sections were incubated overnight with goat anti-DDAH-1 antibody (1:100; ab2231, Abcam) in PBS-T-Azide (0.1 M PBS + 0.3% Triton X100 + 0.002% Sodium Azide) at RT, followed by donkey anti-goat Alexa Fluor™ 488 secondary antibody in PBS-T (1:1000; Invitrogen, CA, USA) for 2 hrs. Following washes in PBS, sections were mounted in Vectashield medium (Vector Laboratories, CA, USA).

Double immunofluorescent staining of DDAH-1 with NeuN, CGRP, IB4, β-III Tubulin and NF200

Sections were incubated overnight at RT in goat anti-DDAH-1 antibody (1:100; ab2231 Abcam) plus one of the following primary antibodies/lectin: mouse anti-NeuN (1:1000; VMA377, Abcys, Paris, France), IB4-FITC (1:700; L2895, Sigma Aldrich, MO, USA), rabbit anti-CGRP (1:4000; Sigma Aldrich, MO, USA), mouse anti-β-III Tubulin (1:1000; Promega, Charbonnieres, France) or mouse anti-NF200 (1:1000; N0142, Sigma Aldrich, MO, USA). After washing in PBS, tissues were incubated for 2 hrs at RT in a mixture of secondary antibodies: donkey anti-goat Alexa Fluor™ 568 (1:1000) and donkey anti-mouse Alexa Fluor™ 488 (1:1000). Tissues were washed in PBS and mounted in Vectashield medium (Vector Laboratories, CA, USA).

Specificity of DDAH-1 immunostaining

Goat anti-DDAH-1 primary antibody (1:100; ab2231 Abcam) was pre-absorped with 10x excess (weight/weight) of the peptide used to generate the antibody (ab99047, Abcam) overnight at 4°C. The mixture was then centrifuged for 20 min at 10000 rpm and the top half of the solution was collected and applied to tissue sections. A positive control (antibody alone at the same dilution) and a negative control (lack of primary antibody) were run in parallel.

In vivo Electrophysiology – Set-up

Rats were anesthetized using 4-5% isoflurane (66% N_2O & 33% O_2) and a tracheal cannula was inserted. Rats were placed in a stereotaxic frame and core body temperature was maintained at 37°C using a feedback heating blanket. Anesthesia was reduced to 2.5% isoflurane and a laminectomy was performed at the L1-L3 vertebral level to expose the L4-L5 segments of the spinal cord. Anesthesia was then maintained at 1.5% isoflurane.

Extracellular recordings from single convergent deep dorsal horn (> 600 µm) wide dynamic range neurons (WDRs) were made using parylene coated tungsten electrodes (A-M Systems, Sequim, WA, USA). WDR neurons respond to both innocuous and noxious stimulation in a graded manner, and can respond to mechanical, thermal, electrical and chemical stimuli. Data was captured by a CED 1401 interface coupled to a Pentium computer with Spike 2 software (Cambridge Electronic Design, UK; PSTH and rate functions).

Electrical stimulation of the rat hindpaw to induce wind-up

A train of 16 transcutaneous electrical stimuli (2 ms wide pulses, 0.5 Hz) was applied at 3x the threshold current for C-fibers via two stimulating needles inserted under the skin of the hindpaw. A post-stimulus histogram was constructed and Aβ- (0-20 ms), Aδ- (20-90 ms) and C-fiber (90-300 ms) evoked responses were separated and quantified by latency. Responses occurring after the C-fiber latency band were taken to be the post-discharge (PD) of the cell (300-800 ms). Input (non-potentiated response) was calculated as: response after the first stimulus × 16. Wind-up was calculated as: (total number of action potentials after 16 stimuli) – (Input).

Following stable control responses at 10 min intervals, L-291 was injected directly onto the surface of the spinal cord (after removal of residual cerebrospinal fluid) in cumulative doses

of 1.2, 12 and 120 µg in a volume of 50 µl (0.1 mM, 1 mM and 10 mM respectively) using a Hamilton syringe. Electrical tests were continued at 10 min intervals with the effect of each dose followed for 1 hr. Control values were obtained by averaging the responses of the three neuronal tests immediately prior to initial drug administration. A separate group of animals received 120 µg (10 mM) of the control L-456 for comparison.

Neuronal Formalin Test

WDR neurons were characterized prior to formalin administration. Transcutaneous stimulating needles delivered electrical stimuli to the receptive field. Next, thermal stimulation was applied using a constant jet of water onto the centre of the receptive field to indicate a strong C-fiber input to the WDR neuron , which is required for the response to subcutaneous formalin [8].

Rats were then pre-treated by topical spinal administration of 12 µg of L-291 (1 mM) or 120 µg of L-456 (10 mM), 20 min prior to the injection of formalin. Both drugs were delivered in a volume of 50 µl using a Hamilton syringe. Formalin (5%, 50 µl) was prepared from a 37% formaldehyde solution and injected subcutaneously into the hindpaw receptive field. Firing responses of WDR neurons were recorded for 70 min. Activity was displayed as a rate recording and quantified in 10 min time bins.

Behavioral Formalin Test

Rats (220-250 g) were placed in a Plexiglas box and acclimatized for 30 min. Next, rats were lightly anesthetized with isoflurane and injected intrathecally with 20 µl of either 24 µg of L-291 or 24 µg of L-456 (both 5 mM) using a 25-gauge needle and syringe inserted into the subarachnoidal space between the L5 and L6 spinal processes. Experimenters were blind to treatment for the whole testing period. Twenty minutes later rats received a subcutaneous injection of 50 µl of formalin (5%) into the plantar surface of the right hindpaw. The time

spent lifting, flinching, licking and biting the injected paw was recorded in seconds (s) during the 60 min period following formalin administration. Data was captured in 5 min time bins.

Statistical Analysis

Effects of L-291 on nitrite accumulation and following hindpaw electrical stimulation were compared using one-way repeated measure ANOVA, followed by Bonferroni multiple comparison post-tests. Cell characteristics were compared between treatment groups by Student's unpaired t-tests. Formalin time courses were compared between treatment groups by two-way repeated measure ANOVA, followed by Bonferroni post-tests. Total activity in the first and second phases was compared by quantifying the area under each curve and analyzed by one-way ANOVA, followed by Bonferroni post-tests. Statistical analyses were conducted using GraphPad Prism v.4 software (GraphPad Software, San Diego, La Jolla, CA).

RESULTS

DDAH-1 is found in sensory neurons within the dorsal root ganglia (DRG) and spinal dorsal horn

Western blot analysis revealed DDAH-1 protein expression in rat hippocampus, DRG and the dorsal horn (Figure 1a). DDAH-1 immunostaining in the dorsal horn was present in scattered neuronal profiles in all laminae (Figure 2a). The intensity of staining was relatively weak, though neurons in the lateral spinal nucleus (LSN) were more strongly stained (Figure 2b). Double staining with NeuN, a marker of neuronal nuclei, did not show overlap with DDAH-1 but rather DDAH-1 staining was observed around NeuN, suggesting that DDAH-1 is located in neuronal soma (Figure 2c). In the DRG, DDAH-1 immunoreactivity was observed in neurons of all sizes (Figure 2e), as confirmed by double staining with the neuronal marker β-III Tubulin (Figure 2g, 100% of β-III Tubulin neurons were DDAH1 positive). Levels of expression varied between different DRG neurons as demonstrated by the strong colocalization of DDAH-1 in large-sized myelinated neurons (Figure 2h, 100% of NF 200 positive neurons were DDAH1 positive) and small-sized peptidergic neurons (Figure 2j) but relative absence in small-sized non-peptidergic neurons (Figure 2i). Double staining with IB4, a marker of small sized non peptidergic nerons showed that only 11.5% of IB4 positive neurons expressed DDAH1. Double staining with the neuropeptide CGRP, showed that a large proportion (76.9%) of CGRP positive neurons expressed DDAH1. Specificity of the staining was confirmed by pre-incubation of the primary antibody with a DDAH-1-specific peptide that prevented all staining in spinal cord and DRG (Figure 2d,f).

Selective DDAH-1 inhibitor L-291 reduces nitric oxide synthesis in sensory neurons

To demonstrate a functional role for DDAH-1 in NO production in sensory neurons, we applied L-291 to cultured DRG neurons and measured nitrite accumulation, a marker for NO synthesis. L-291 caused a significant and concentration-dependent reduction in nitrite levels

(Figure 1b) consistent with a concentration dependent increase in ADMA levels (Figure 1c). Our results confirm the presence of DDAH-1 in sensory neurons of the DRG and spinal cord and suggest that DDAH-1 contributes to NO signaling in neurons.

Spinal L-291 inhibits C-fiber-evoked responses, post-discharge and wind-up of deep dorsal horn wide dynamic range (WDR) neurons

We next assessed the function of DDAH-1 in sensory and nociceptive processing in the dorsal horn. Spinal administration of L-291 (1.2 μg, *n* = 9; 12 μg, *n* = 9; 120 μg, *n* = 6) produced selective and significant dose-dependent inhibition of C-fiber-evoked responses (Figure 3c), post-discharge (Figure 3d) and wind-up (Figure 3f,g) of WDR neurons. No changes were seen in Aβ-fiber- or Aδ-fiber-evoked responses (Figure 3a and Figure 3b, respectively), nor in input (Figure 3e). The control drug, L-456 (120 μg, *n* = 8), had no effect (Figure 3a-h). Peak inhibitory effects of L-291 were evident by 40 min post-administration and persisted for the remainder of the recording period. Pre-drug control responses did not differ between treatment groups (Supplementary Table 1).

Thus, spinal application of L-291 selectively reduces C-fiber-evoked responses, post-discharge and NMDA-dependent wind-up of WDR neurons, indicating a role for DDAH-1 in nociceptive signaling and plasticity within the dorsal horn rather than basal sensory transmission. No changes were seen in A-fiber-evoked responses and input, suggesting a predominantly postsynaptic effect of L-291 and DDAH-1 inhibition.

Formalin-induced central sensitization of deep dorsal horn WDR neurons is reduced by spinal pre-treatment with L-291

In electrophysiological recordings in rats pre-treated spinally with control drug L-456 (120 μg, *n* = 14), formalin injection into the hindpaw induced a characteristic biphasic neuronal firing response (Figure 4a-c). In comparison, spinal pre-treatment with L-291 (12 μg, *n* = 10)

significantly and selectively reduced second phase neuronal firing (Figure 4a,b,d). No change in first phase activity was observed. Neurons were characterized prior to injection of drugs and were comparable between treatment groups (Supplementary Table 2).

Spinal L-291 inhibits pain-related behaviors due to formalin-induced central sensitization

Effects of spinal inhibition of DDAH-1 were next assessed in the formalin behavioral test. Pain-related behaviours were divided into two categories: reflexive lifting and flinching behaviours; and active licking and biting of the injured paw. In rats pre-treated intrathecally with the control compound L-456 (24 μg, n = 6), formalin induced a biphasic pain-related behavioral response (Figure 4e,f). In contrast, pre-treatment with L-291 (24 μg, n = 6) significantly and selectively decreased pain-related behavior during the second phase (Figure 4e,f). No difference was seen during the first phase between treatment groups. The inhibitory effect of L-291 during the second phase of the response to formalin reduced both types of behavior (Supplementary Figure 2).

Thus, spinal administration of L-291 reduces both second phase neuronal firing and pain-related behaviors induced by intraplantar formalin injection, suggesting that DDAH-1 has a role in NMDA-dependent central sensitization of deep dorsal horn WDR neurons.

DISCUSSION

The results of this study suggest that DDAH-1, an enzyme that metabolizes asymmetric methylarginines, contributes to NMDA-dependent plasticity of spinal dorsal horn sensory neurons following noxious peripheral stimulation. We have demonstrated the presence of DDAH-1 protein in the spinal cord and DRG, and, importantly, that pharmacological inhibition of DDAH-1, with a selective inhibitor, L-291, reduces NO synthesis, neuronal hyperexcitability and pain-related behavior following formalin-induced spinal central sensitization.

Biochemical analyses demonstrated protein expression of DDAH-1 in the soma of DRG and dorsal horn neurons. In the DRG, expression tended to be higher in larger myelinated cell bodies but was also visualized in small, probably nociceptive, peptidergic cells. In the dorsal horn, cell bodies and axons were stained in Lateral Spinal Nucleus (LSN), reinforcing the idea that DDAH-1 is found in both afferent and intrinsic spinal neuronal systems. Interestingly the LSN receives deep inputs from tissue suggesting a role of ADMA in their regulation as well as the cutaneous evoked activity in the present study.

Despite strong expression in large myelinated cells, A-fiber-evoked responses and input were unaffected by L-291, suggesting that DDAH-1 in these cells may not contribute to normal sensory signalling.

Spinal administration of L-291 inhibited post-discharge and wind-up of WDR neurons induced by electrical stimulation of the hindpaw. C-fiber-evoked responses were also reduced, though to a lesser extent. A-fiber-evoked responses and input were unaffected by L-291. These selective inhibitory effects of spinal DDAH-1 antagonism on NMDA-mediated neuronal events suggest a predominantly postsynaptic effect and are similar to those produced by spinal inhibition of nNOS [31; 33], as well as drugs targeting spinal NMDA receptors[7; 6] Targeting DDAH-1 may be a novel way of modulating the NMDA-NO

signaling pathway through manipulation of an endogenous control, which may allow physiopathological signaling to be selectively modulated and so potentially increasing tolerability over direct NMDA receptor blockers. The selectivity of L-291 activity also suggests that altered local vascular effects do not influence spinal function, as these would be expected to alter all neuronal responses in a non-selective manner. Indeed, no obvious changes in the central blood vessel shape or size were observed during electrophysiological recordings following administration of L-291.

In both neuronal and behavioral formalin tests, spinal administration of L-291 reduced spinal central sensitization, as shown by a reduction of second phase activity. The lack of any effect on first phase responses suggests that L-291 does not alter basal pain but rather attenuates potentiation of pain transmission. These findings are consistent with those of several studies investigating the role of the NOS pathway in the formalin test. Both systemic and spinal administration of NOS inhibitors selectively reduces second phase pain-related behavior[20; 24; 5] [. Formalin injection into the hindpaw has been shown to upregulate nNOS in the dorsal horn [13; 16], while formalin-induced c-Fos expression in the superficial dorsal horn is reduced by spinal NOS inhibition [28]. These studies establish a significant contribution of NO and NOS to spinal central sensitization and provide a context for the effects of DDAH-1 inhibition, which indirectly suppresses NO synthesis [19]. Effects of L-291 on neuronal activity and pain-related behavior were produced at similar drug concentrations as effects on NO synthesis in DRG neurons.

This study is the first to demonstrate protein expression of DDAH-1 in the CNS and to suggest a functional role for DDAH-1 in neuronal signaling, as shown by reduction of NO synthesis in DRG neurons and modulation of NMDA-dependent spinal nociceptive plasticity by the DDAH-1 inhibitor L-291. DDAH-1 may therefore be a novel analgesic target in chronic pain. Furthermore, DDAH-1 inhibitors such as L-291 may prove to be effective therapies in various neuronal disorders where nNOS activity and NO synthesis is elevated and causally

linked to the underlying pathology, though consideration must be given to potential cardiovascular side-effects.

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FIGURE LEGENDS

Figure 1. Expression of DDAH-1 in the dorsal root ganglion (DRG) and dorsal horn, and effect of DDAH-1 inhibitor L-291 on nitric oxide production in DRG neurons

(a) Western immunoblot showing expression of DDAH-1 protein in hippocampus (two left lanes), DRG (two middle lanes) and spinal dorsal horn (two right lanes). β-III Tubulin was used as a loading control. Measurement of (b) nitrite production or (c) ADMA levels in isolated DRG neurons treated with L-291 (0-10 mM) (BLD indicates values fall below the limit of detection). Data expressed as mean nitrite concentration (± s.e.m.) of 4-10 wells from 3-5 animals; $*$ $p < 0.05$, $**$ $p < 0.01$ and $***$ $p < 0.001$ versus control (0 mM L-291), one-way repeated measure ANOVA followed by Bonferroni post-hoc tests or Student's paired T-Test.

Figure 2. DDAH-1 is expressed in sensory neurons within the DRG and dorsal horn

(a-j) Expression of DDAH-1 in the rat spinal cord and dorsal root ganglion. (a) Immunofluorescent staining of DDAH-1 in the lumbar spinal dorsal horn is localized in weakly stained neuronal soma in the grey matter. (b) Neurons stained in the lateral spinal nucleus (LSN) display stronger staining in the dorsal horn. (c) Merged double staining image of DDAH-1 (red) and NeuN (green, marker of neuronal nuclei) in the dorsal horn. (d,f) Specificity of the anti-DDAH-1 antibody demonstrated by pre-absorption with DDAH-1 peptide and subsequent loss of staining in (d) spinal cord and (f) DRG. (e) Immunofluorescent staining of DDAH-1 in DRG neurons. (g-j) Merged double staining images of DDAH-1 (red) in DRG with (g) β-III Tubulin (green, neuronal marker); (h): with NF200 (green, marker of large myelinated DRG neurons); (i) with IB4 (green, marker of small non-peptidergic DRG neurons); (j) with CGRP (green, marker of small peptidergic DRG neurons). Scale bar = a: 70 μm, b: 78 μm, c-d: 28 μm, e-f: 70 μm, g: 42 μm, h: 55 μm, i: 97 μm, j: 61 μm.

Figure 3. Spinal L-291 decreases C-fiber evoked responses and postdischarge of WDR neurons

(a-h) Effect of spinal application of L-291 (1.2 µg (0.1 mM), *n* = 9; 12 µg (1 mM), *n* = 9; 120 µg (10 mM), *n* = 6) or L-456 (120 µg, *n* = 8) on (a-c) afferent-evoked responses, (d) postdischarge, (e) input and (f) wind-up of WDR neurons, induced by transcutaneous electrical stimulation of the hindpaw. White bars represent pre-drug control. Data presented as mean ± s.e.m. of pre-drug control responses; ∗ $p < 0.05$, ∗∗ $p < 0.01$ versus pre-drug control, one-way repeated measure ANOVA followed by Bonferroni post-tests. (g,h) Examples of wind-up of single WDR neurons following repetitive electrical stimulation in the presence of spinal (g) L-291 (1.2 µg and 12 µg) or (h) L-456 (120 µg).

Figure 4. Spinal L-291 reduces formalin-induced central sensitization

(a) Time course of WDR firing response to subcutaneous formalin (5%, 50 µl) injection into the hindpaw following spinal pre-treatment with control L-456 (120 µg/10 mM *n* = 14) or L-291 (12 µg/1 mM, *n* = 10; ∗∗ $p < 0.01$ at 50 min, ∗∗∗ $p < 0.001$ at 60min, ∗∗∗ $p < 0.001$ at 70min versus L-456; two-way repeated measure ANOVA followed by Bonferroni post-tests). (b) Total neuronal activity during the 1st (0-10 min) and 2nd phases (10-70 min) of the formalin response with spinal L-456 or L-291 (2nd phase: ∗∗∗ $p < 0.001$ versus L-456, one-way ANOVA followed by Bonferroni post-tests). (c,d) Representative rate recordings of firing responses of WDR neurons to formalin following spinal pre-treatment with (c) L-456 or (d) L-291. (e) Time course of pain-related behaviors induced by intraplantar injection of formalin (5%, 50 µl) following intrathecal pre-treatment with L-456 (24 µg) or L-291 (24 µg, ∗ $p < 0.05$ at 35 min versus L-456; two-way repeated measure ANOVA followed by Bonferroni post-tests). (f) Total pain-related behavior during the 1st phase (0-10 min) and 2nd phase (10-60 min) of the response to formalin following intrathecal pre-treatment with L-456 or L-291 (2nd phase: ∗∗ $p < 0.01$ versus L-456; one-way ANOVA followed by Bonferroni post-tests). All data presented as mean ± s.e.m., *n* = 6 in each group.